IMPROVED METHOD OF PERFORMING GENETIC ANALYSES ON
REPRODUCTIVE TRACT CELL SAMPLES

1   TITLE

Improved method of performing genetic analyses on female reproductive tract cell
5   samples.

2   FIELD OF THE INVENTION

This invention relates to methods of performing genetic analyses on cell samples
taken from the reproductive tract of animals. It relates further to combinations of
isolation/enrichment of cells from samples, the genetic identification and analysis of such
10   cells and the automated processing of resultant data to enable high throughput genetic
analysis and thus application in clinical practice. More particularly, this invention relates to
high throughput enrichment of fetal cells from cervical samples, and in particular, Pap
smears. In a particular form, this invention relates to use of fetal cells enriched from
cervical samples for subsequent genetic analysis such as Down syndrome, sex and
15   single gene defects and blood group testing. Particular embodiments of this invention
utilize cell isolation procedures such as laser micro-dissection, magnetic and/or
fluorescent activated cell sorting, singly and in combination, to enrich fetal cells to levels
of purity that readily enable nucleic acid isolation for genetic analysis. Particular
embodiments of this invention utilize techniques such as nucleic acid amplification and/or
20   a variety of analysis techniques such as multiplex PCR to subsequently genetically
analyse pooled or single cells.

3   BACKGROUND OF THE INVENTION

Genetic defects are *the* major cause of embryonic, fetal and neonatal death as
well as being responsible for a large proportion of childhood disabilities. The life-long cost
25   of these disabled children to society is enormous. Although many defects are detected by
the annual ~50,000 prenatal tests in Australia, tests are only offered to high-risk mothers
as they are invasive (~1% risk of miscarriage) and/or expensive. One result of current
screening strategies is that the vast majority of babies with genetic defects are born to the
low-risk population. Currently prenatal diagnosis of chromosomal and single gene
30   disorders requires the withdrawal of fetal cells from the uterine cavity by invasive
procedures such as amniocentesis or chorionic villus sampling (CVS). These techniques,
although highly reliable, carry procedurally related risks such as miscarriage (0.5-1%),
require a high level of technical expertise, can take several weeks for results and can only

be performed relatively late in pregnancy. The miscarriage risk and high cost limit availability limits the availability of prenatal diagnosis to high risk mothers only.

Current prenatal risk assessment has two main limitations. Firstly, high risk mothers are offered tests even though most (~95%) do not have an affected fetus, 5 resulting in unnecessary miscarriage risk and high cost. Secondly, affected children from low risk groups are usually not identified.

Currently prenatal diagnosis of chromosomal and single gene disorders requires the extraction of fetal cells from the uterine cavity by invasive procedures such as amniocentesis or chorionic villus sampling (CVS). These techniques, although highly 10 reliable, carry procedurally related risks such as miscarriage (0.5-1%), require a high level of technical expertise, take several weeks for results and can only be performed relatively late in pregnancy. Thus, they are only offered to women considered at high risk due to age, genetic history or other indicative factors.

One less invasive alternative approach is to use maternal blood as a source of 15 fetal cells for which many fetal cell enrichment methods have been developed, for example as described in United States Patent 5,629,147, United States Patent 5,646,004 and International Publication WO 98/02528.

However major technical difficulties remain due to the extremely low numbers of fetal cells found in the maternal circulation, the extreme difficulties in isolating such cells, 20 the positive identification of fetal cells and the presence of fetal cells from previous pregnancies which may confound identification and diagnosis.

The presence of fetal cells in the endocervical canal was first published in Shettes, 1971, Nature **230** 52. Since then there have been many studies confirming the presence of fetal cells with varying success during the first trimester (Fejgin et al., 2001, Prenatal 25 Diagnosis **21** 619; These studies all confirm that the number of fetal cells present in the endocervical canal of pregnant women is extremely low and secondly that these cells are difficult to isolate.

Accordingly, the value of maternal cervical samples as a source of fetal cells for genetic analysis has remained controversial. Additionally, there have been major 30 concerns as to the invasiveness and safety of cervical sampling and the practicality of using cervical samples as a source of relatively low abundance fetal cells. Indeed, Overton et al., 1996, J. Am. Obstet. Gynecol. **175** 382 concluded that fetal cells cannot be obtained from the endocervix by minimally invasive techniques in sufficient yield for

prenatal genetic diagnosis.

More recently Cioni *et al.*, 2003 Prenatal Diagnosis **23** 168-171 confirmed that fetal cells were not detected in a consistent and reliable fashion and therefore such sampling techniques cannot be regarded as a promising tool towards minimally invasive
5 prenatal diagnosis.

It has been shown (Kingdom et al., 1995 Obstetrics and Gynaecology, 86 pp 283-288) that fetal cells can be isolated from cervical samples such as PAP smears using fluorescently labelled antibodies to remove maternal cells (negative enrichment) and extract fetal cells (positive enrichment) with subsequent genetic analysis in a similar
10 manner to that performed for fetal cells in maternal blood, Immunology, 30 (2-3) pp.194-201; Durrant et al., 1996 British Journal Of Obstetrics And Gynaecology, 103, (3), 219-222). However such techniques are expensive, time intensive and are limited in sample throughput to <10 samples per day, which is insufficient for the high throughput required for cost-effective clinical application. For the purposes of this invention, high throughput
15 refers to the ability to process in excess of 50 samples per 24hr day.

Previous work has identified five main difficulties in applying performing genetic analysis from cervical cells at a high throughput level sufficient for practical application.

1.   Obtaining sufficient cells. Attempts to obtain fetal cells from the cervix of pregnant women have been hampered by the need to retrieve the large number of cells
20 required for genetic diagnosis. Although this requirement has been partially overcome by no longer requiring large numbers of cells due to recent advances such as multiplex fluorescent PCR which now allow multiple genetic analyses from single cells, single cell multiplex PCR remains a highly technical process practiced by very few laboratories worldwide due to its high complexity. Previous work has indicated that fetal cells are not
25 detected in a consistent and reliable fashion and therefore cannot be regarded as a promising tool for prenatal analysis.

2.   Isolation of fetal cells from the sample. Recent results suggest that fetal cells can be isolated and diagnosed in only ~22% of cases due to the presence of "contaminating" maternal cells. Previous approaches have generally concentrated on
30 isolating cervical cells by morphology or cell sorting. Unfortunately, morphology grading is extremely time-consuming, expensive and generally unreliable and inaccurate. Cell sorting such as FACS or MACS has been generally unspecific resulting in either major

maternal contamination and misdiagnosis or insufficient fetal cells. This remains a major limitation to practical application.

3.   Genetic identification of cells to determine fetal source. It is essential to identify the isolated cells as being fetal to avoid misdiagnosis from maternal cells. Although single cell DNA fingerprinting techniques such as single cell multiplex PCR can be used to forensically identify the source of a single cell, again single cell multiplex PCR remains a highly technical process practiced by very few laboratories worldwide due to its high complexity. However results from fetal cells were not detected in a consistent and reliable fashion and therefore cannot be regarded as a promising tool.

4. Genetic diagnosis from small cell numbers. Diagnosis from single or low numbers of cells is extremely difficult. Fetal cells have been identified in cervical samples mainly by the identification of male cells within the sample, aneuploidy screening (the primary reason for prenatal diagnosis) cannot usually be performed nor diagnosis made if the fetus is female. This requirement has been partially overcome by recent advances such as multiplex fluorescent PCR which now allow multiple genetic analyses from single cells. However again results from fetal cells were not detected at a consistent and reliable enough level to be considered as a promising tool towards minimally invasive prenatal diagnosis.

5.   Sample collection. The most recent work on PAP smears have utilised so called "thick section" PAP smears. However clinical PAP smears collections are moving towards a "thin section" smears which have not previously been used for fetal cell isolation.

Each of the five major difficulties mentioned above have singly, and particularly in combination, prevented practical application of non-invasive prenatal diagnosis.

However, by improving current methods and developing new methods in combination, we have invented a novel high throughput method, to rapidly and efficiently accurately target and genetically diagnose affected babies in both high-risk mothers and previously designated low risk pregnancies. For the first time non-invasive prenatal diagnosis is now a practical application and will have enormous health, social and economic benefit.

4   SUMMARY OF THE INVENTION

Notwithstanding the prior art teaching that cervical samples are very poor sources of fetal cells for genetic analysis, the present inventors have developed improvements to a variety of techniques which individually, and particularly in

5   combination, improve the scope for providing an efficient method encompassing reliable and efficient combination of methods which isolate and enrich fetal cells from cervical samples, performed genetic analysis (DNA fingerprinting and/or genetic diagnosis/screening) with subsequent data analysis, which enables sufficient high- throughput processing for practical application.

10   Accordingly, the invention generally provides a method of analysing the genetic characteristics of a reproductive tract cell sample taken from a subject, the method comprising the steps of:

(a)   taking a cell sample from the subject

(b)   isolating one or more target cells from the sample; and

15   (c)   analysing the genetic characteristics of the one or more target cells

isolated from the sample.

Preferably, the subject is a mammal.

In a preferred form of the invention, the subject is a human being. In an alternative embodiment of the invention, the subject is a non-human mammal.

20   Preferably, the one or more target cells sought to be analysed via the use of the method are of embryonic origin. Whenever used in this specification, the term "embryo" (or "embryonic") refers to an organism prior to birth and the expression includes all gestational stages (including fetal stages). It is particularly preferred that the one or more target cells are or comprise fetal cells.

25   Preferably, step (a) of the method comprises a non-invasive or substantially non-invasive procedure.

It is particularly preferred that if the cell sample is taken by using an endocervical brush or a cytobrush to gather a cell sample from the subject by scraping the lining of the cervix or reproductive tract. Preferably further, the reproductive tract cell sample is:

(a)   a cervical cell sample; or

(b)   a high vaginal smear.

It is especially preferred that the reproductive tract cell sample is taken via a Pap smear procedure. A Pap smear procedure suitable for use in the method may be either:

5   (a)   a thin section Pap smear procedure; or

(b)   a thick section Pap smear procedure.

Where the subject is a human being, it is particularly preferred that the reproductive tract cell sample is obtained via a Pap smear procedure performed at between 5 and 31 weeks gestation. In that event, it is especially preferred that the method 10   is performed in the first or second trimester of gestation.

The cervical cell sample may additionally comprise one or more of the following:

(a)   blood; and

(b)   vaginal cells.

Preferably, in use, step (b) of the general method of the invention comprises an 15   enrichment procedure, to isolate one or more target cells from other cells present in the reproductive tract cell sample.

It is further preferred that the enrichment procedure comprises either or both:

(a)   a positive enrichment procedure, whereby one or more target cells are extracted from the cell sample by differentiation according to antigen 20   expression (or non-expression) in those cells; and

(b)   a negative enrichment procedure, whereby one or more non-target cells are differentiated from target cells by using identification means and the non-target cells are then removed from the reproductive tract cell sample.

Preferably further, the enrichment procedure comprises the use of one or more of 25   the following techniques to differentiate target cells from non-target cells in the reproductive tract cell sample:

(a)   exploitation of differences in physical cell characteristics;

(b)   exploitation of biological differences between cell types; and

(c)   exploitation of genetic differences between cell types.

Preferably further, the enrichment procedure comprises a differentiation step and the differentiation step comprises the use of one or more of the following:

(a)   exploiting physical differences between cells in the reproductive tract cell sample;

(b)   exploiting differences in morphological characteristics as between cells in the reproductive tract cell sample;

(c)   exploiting differences in the granularity characteristics as between cells in the cervical cell sample;

(d)   exploiting differences in DNA content as between cells in the reproductive tract cell sample ;

(e)   density separation ;

(f)   fluorescence activated cell sorting;

(g)   magnetic activated cell sorting ;

(h)   cell lysis;

(i)   complement-mediated lysis;

(j)   flow cytometry;

(k)   panning;

(l)   charge-flow separation and/or

(m)   laser microdissection.

In an alternative embodiment, the enrichment procedure comprises a differentiation step which involves the use of one or more of the following:

(a)   exploiting immunological differences between target and non-target cells in the reproductive tract cell sample; and/or

(b)   cell culture methods that promote selective propagation of the cells sought to be isolated.

Preferably further, the enrichment procedure used comprises the exploitation of immunological differences between the target and non-target cells in the reproductive tract cell sample, and those cells are differentiated by the use of at least one:

(a)   antibody that binds a target cell antigen; and/or

(b)   antigen that binds to an antibody (or an antigen-antibody complex) on or in a target cell

in the reproductive tract cell sample.

It is especially preferred that an antibody is used to bind to antigens on or in the one or more target cells contained in the reproductive tract cell sample. In this embodiment of the invention, it is particularly preferred that the antibody is capable of binding to one or more embryonic cell antigens. Preferably further, the antibody is capable of binding to one or more of.

(a)   PAX-8;

(b)   CD71;

(c) γ globin (fetal);

(d) ζ globin (embryonic);

(e)   glycophorin A;

(f)   CD 36;

(g)   Fkl-1;

(h)   EPO-R;

(i)   CDw50;

(j)   CD45;

(k)   Human chorionic gonadotrophin;

(l)   Placental alkaline phosphatase;

    (m)   Human placental lactogen;

    (n)   · Folate binding protein (LK26); or

    (o)   a HLA antigen.

Preferably further, in the use of the method of the invention, the genetic analysis

5   of the one or more target cells isolated from the reproductive tract cell sample comprises the use of either or both:

    (a)   genetic amplification techniques; and/or

    (b)   genetic identification techniques.

Preferably, the target cells to undergo genetic analysis are such that the nucleic

10 acid(s) are contained within the target cell. In an alternative embodiment however, the method also comprises the step of isolating one or more cell nuclei from the target cell (or cells) and the subsequent selection of one or more isolated nuclei.

Preferably further, where a genetic amplification technique is used, the amplification technique comprises or utilises one or more of the following:

15   (a)   polymerase chain reaction;

    (b)   comparative genome hybridisation;

    (c)   single nucleotide polymorphism genotyping;

    (d)   fluorescent in situ hybridisation; .

    (e)   whole genome amplification;.and/or

20   (f)   · rolling circle amplification, and/or

    (g)   Linear amplification.

Preferably further, the techniques (a) to (f) are carried out:

sequentially, with technique (a) being used first; or in any other sequence.

Preferably further, where the method comprises the use of a genetic identification

25 technique, the genetic identification technique comprises or utilises one or more of the following:

      (a)   DNA fingerprinting

      (b)   Nucleic acid separation techniques;

      (c)   polymerase chain reaction;

      (d)   multiplex polymerase chain reaction;

5   (e)   comparative genome hybridisation;

      (f)   single nucleotide polymorphism genotyping;

      (g)   fluorescent in situ hybridisation;

      (h)   reverse transcriptase-polymerase chain reaction;

      (i)   whole genome amplification;.and/or

10   (j)   rolling circle amplification.

In the performance of the method of the invention, these techniques may be carried out:

      (a)   sequentially in the order listed, with first named technique being used first; or

15   (b)   in any other sequence.

Preferably further, in the performance of the method, the results of the performing the method are analysed.

In order to improve the efficiency of the performance of the method, preferably, the performance of at least one of the steps comprising the method is automated or semi-

20 automated.

Preferably further, the method is used to identify or diagnose the presence of at least one predetermined genetically mediated. condition in the one or more target cells contained in or isolated from the reproductive tract cell sample.

The predetermined genetically mediated condition may comprise one or more of

25 . the following:

      (a)   Genetic syndromes;

(b)   Cystic fibrosis;

(c)   Factor V Leiden mutation;

(d)   Human A blood grouping;

(e)   Human B blood grouping;

5   (f)   Human O blood grouping;

(g)   Human Rh positive blood factor grouping;

(h)   Human Rh negative blood factor grouping;

(i)   Tay Sachs disease

(j)   Fabry disease

10   (k)   Haemachromatosis

(l)   Haemoglobinopathies

(m)   The DNA fingerprint of an embryo borne by the subject; and

(n)   The sex of an embryo borne by the subject.

It will be appreciated however, this list is not exhaustive.

15   Preferably further, each genetic condition can be assessed individually, sequentially or simultaneously with any other genetic condition or combination of other genetic conditions.

It is particularly preferred that the use of the method leads to improved turn around times for receiving the results of analysis of cell samples. In this context, it is preferred

20   that the time between:

(a)   collection of the reproductive tract cell sample; and

(b)   receiving the results of analysis of the reproductive tract cell sample

is between six hours and seven days.

It is further preferred that the time between:

(a)   collection of the reproductive tract cell sample; and

(b)   receiving the results of analysis of the reproductive tract cell sample

is within 48 hours from the time of collecting the reproductive tract cell sample.

More preferably, the time between:

5   (a)   collection of the reproductive tract cell sample; and

(b)   receiving the results of analysis of the reproductive tract cell sample

is within 24 hours from the time of collecting the reproductive tract cell sample.

Even more preferably, the time between:

(a)   collection of the reproductive tract cell sample; and

10   (b)   receiving the results of analysis of the reproductive tract cell sample

is within 6 hours from the time of collecting the reproductive tract cell sample.

It is also preferred that the use of the method will lead to improvements in the number of analyses that can be carried out within a given time. Preferably, the use of the method of the invention enables an analyst to perform the method at least 10 times in a
15   period of 24 hours from the collection of a first reproductive tract cell sample from a subject.

More preferably, such an analyst is able to perform the method at least 20 times in a period of 24 hours from the collection of a first reproductive tract cell sample from a subject.

20   Even more preferably, the analyst is able to perform the method at least 50 times in a period of 24 hours from the collection of a first reproductive tract cell sample from a subject.

In one aspect then, the invention provides a high throughput method of cell isolation including the step of enriching one or more fetal cells from a cervical sample.

25   In another aspect, the invention also provides a high throughput method of obtaining a nucleic acid sample, including the step of isolating one or more nucleic acids from one or more fetal cells that have been enriched from a cervical sample.

In another aspect, the invention potentially improves the efficiency of whole genome amplification from single cells thus improving the potential for genetic analysis.

In another aspect, this invention improves the robustness of whole genome amplification from single cells by reducing the incidence of allelic dropout, whole locus
5   dropout and preferential amplification, thus improving the potential for genetic, and particularly quantitative, analysis.

In yet another aspect, the invention provides higher throughput semi-automated and automated methods to increase sample throughput and increase cost-effectiveness.

In yet another aspect, the invention provides a high throughput method of genetic
10 analysis including the step of analyzing a nucleic acid obtained from one or more fetal cells that have been enriched from a cervical sample.

In a further aspect, the invention relates to the higher throughput use of one or more fetal cells enriched from a cervical sample for genetic analysis.

In a further aspect, the invention combines improvements over a number of steps
15 (isolation, DNA fingerprinting and genetic analysis and data processing), which combine to give a significantly increased throughput sufficient for practical application.

It will also be appreciated that other analyses also contemplated by the present invention include biochemical analysis, morphological analysis, histology, cytology, cell culture and the like as well as a variety of genetic analyses, including nucleic acid
20 amplification methods such as PCR, CGH (comparative genome hybridization), whole genome amplification, SNPs (single nucleotide polymorphisms), FISH (fluorescent in situ hybridization) and the like.

In a still further aspect, the invention relates to the high throughput use of one or more fetal cells enriched from a cervical sample for the isolation of a nucleic acid sample.

25 In a yet further aspect, the invention relates to high throughout use of a cervical sample for enrichment of one or more fetal cells for the isolation of a nucleic acid sample.

In a still yet further aspect, the invention relates to high throughout use of a cervical sample for enrichment of one or more fetal cells for genetic analysis.

The invention also relates to high throughout enrichment steps described herein to
30 enrich fetal material from cervical samples.

The invention also relates to the high throughout automation of steps described herein to enrich fetal material from cervical samples.

The invention also relates to any combination of isolation/enrichment techniques with any combination of nucleic amplification and/or genetic identification techniques such as DNA identification, and/or genetic diagnosis and/or automated data analysis to allow sufficiently high throughput for practical application.

It will be appreciated by the skilled addressee that any and all of these high throughput techniques can be readily applied to a variety of samples including, but not limited to, blood, vaginal cells, PAP smears (both thin and thick specimens).

As previously explained, in a preferred embodiment, the invention provides a method of fetal cell analysis including any combination of the steps of:

    i.   enriching fetal cells from a Pap smear sample according to physical characteristics such as size, density, morphology and/or granularity, DNA content; and/or

    ii. positively selecting fetal cells from the cells enriched in step (i) and/or in using at least one primary/secondary antibody set that binds a fetal cell antigen.

    iii. Amplification of generic nucleic acid from isolated sample from step (ii) Genetic identification product from step (iii) using techniques such as DNA fingerprinting

    iv. Genetic analysis of product from steps (ii), (iii) and/or (iv) including but not limited to specific genetic analysis methods such as multiplex PCR, SNPs, CGH, FISH, RT-PCT and the like. It will be appreciated that steps (v) and (vi) can be combined into a single analysis procedure

    v. Detection of products from steps (iii), (iv) and/or (v) for example utilising nucleic acid separation technologies such as a DNA sequencer;

    vi. automated data processing to create analysis report from step (vi)

The invention also apprehends the use of mico-fluidic devices in connection with the steps described above.

. Throughout this specification, unless otherwise indicated, the terms "comprise", "comprises" and "comprising" are used as words of inclusion, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

5   5   BRIEF DESCRIPTION OF THE DRAWINGS AND TABLES

In order that the invention may be further understood, preferred embodiments of the invention will now be described by way of example only, and with reference to the . accompanying drawings, in which:

Fig 1   Represents a schematic summary of the method of the invention

Fig 2   (Table 1) This table lists various STR marker sequences suitable for DNA identification and genetic analysis in accordance with the invention

Fig 3   (Table 2) This table lists examples of markers used in the genetic analysis embodiment of the invention discussed in this specification

Fig 4   (Table 3) This table lists examples of markers used in the DNA identification embodiment of the invention discussed in this specification

Fig 5   (Table 4) This table compares the efficacy of various analytical methodologies suitable for use in connection with the method of the invention

Fig 6   Illustrates the effect of various different cell solution filtration techniques on cell sorting, as demonstrated by dot plots generated by the use of the Beckman Coulter EXPO32 analysis software.

Fig 7   Depicts the results of various Single Nucleotide Polymorphism (SNP) reactions, combining two SNPs (RhE and KEL) ·

Fig 8   Depicts the results of various experiments on single Genomiphi . analysis on single cells, demonstrating the effect of incubation with Betaine on the reliability of the results obtained

Fig 9   Depicts the results of single plex SNP reactions for the SNPs KEL
                  and RhE.

6   DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE
        INVENTION

        The present invention provides a variety of methods applicable to genetic analysis
of fetal cells from maternal reproductive tract cell samples, and in particular, from cervical
5   Pap smears. Such methods include the steps detailed previously, comprising cell
isolation, nucleic acid isolation and amplification, genetic identification and analysis and
automated data processing.

        It will also be appreciated that the present invention is applicable to isolation or
enrichment of other cells of non-maternal origin including, but not limited to, embryonic
10   cells, sperm cells and any cells of cytotrophoblast or syncytiotrophoblast origin.

        It should also be appreciated that the present invention is applicable to isolation or
enrichment of other cells of non-maternal origin from a variety of other sources such as
maternal blood, vaginal cells and the like.

        For the purposes of this invention, by "isolated" is meant material that has been
15   removed from its natural state or otherwise been subjected to human manipulation.
Isolated material may be substantially or essentially free from components that normally
accompany it in its natural state, or may be manipulated so as to be in an artificial state
together with components that normally accompany it in its natural state.

        By "enrich", "enriched" and "enrichment" in the context of cell isolation is meant
20   that cells are obtained in a higher frequency of proportion compared to their frequency or
proportion in a starting sample prior to enrichment. In this context enrichment is also
taken to include 100% enrichment where the fetal cell or cells exist in the absence of
maternal cells.

        Suitably, fetal cells are enriched from a reproductive tract cell sample, which
25   preferably, is a cervical sample. Such samples include and encompass any sample
obtained from the endocervix inclusive of endocervical lavage, aspiration, swabbing;
cytobrush samples; transcervical samples (TCCs) and Pap smears

Preferably, the cervical sample is a Pap smear.

As used herein, a Pap smear is a biological sample comprising one or more cells collected, obtained as a scraping from the cervix.

Typically, a metal or plastic instrument such as a speculum is placed in the vagina
5   to allow visualization of the interior of the vagina and the cervix. A sampling instrument such as a small wooden spatula is used to scrape the outside of the cervix and thereby obtain the cervical sample.

· For the purposes of cervical cancer screening, the scrapings are placed on a glass slide and used for microscopic examination to detect changes in the cells of the cervix.
10 Pap smears are a routine and safe screening procedure to find early warning signs of cervical cancer. The present invention provides a new use of Pap smears as a source of fetal cells for enrichment and subsequent analysis.

In the context of the present invention, said one or more cells typically comprises maternal cells and fetal cells.

15   For the particular purpose of fetal cell isolation, it is preferred that the Pap smear is obtained at·between 5 and 31 weeks gestation.

Cell enrichment may be performed by one or more high throughput cell isolation methods including density separation, complement-mediated lysis, flow cytometry, magnetic bead separation, panning, charge flow separation, laser microdissection and
20 cell culture methods that promote selective propagation of cells to be enriched.

Each cell enrichment method may be performed alone or in combination with one or more other methods to thereby achieve a desired level of cell enrichment or purity.

Additional treatments may be utilized that facilitate cell isolation and enrichment, for example in one embodiment protease treatment (e.g. trypsin digestion) of cervical
25 samples may be performed prior to density gradient enrichment. ·

6.1   Cell enrichment/isolation

It will be appreciated that fetal cell enrichment may be achieved using physical characteristics such as size, density, DNA content, granularity and/or using antibodies directed to fetal antigens not expressed, or expressed at low levels, by maternal cells.

Alternatively, fetal cells may be enriched by virtue of their non-expression of maternal or non-fetal antigens.

Accordingly, fetal cell enrichment may be performed by negative depletion of maternal cells and/or positive selection of fetal cells according to antigen expression.

5   Antigens that may be applicable to antibody-based enrichment include, but are not limited to, PAX-8, CD71, γ globin (fetal) and ζ globin (embryonic), glycophorin A, CD36, Fkl-1, EPO-R, CDw50, CD45, human chorionic gonadotrophin (HCG), placental alkaline phosphatase, human placental lactogen, folate binding protein (LK26) and HLA antigens such as HLA-Class II, for each of which specific antibodies are readily available.

10   Preferred antigens are human placental lactogen, placental alkaline phosphatase, human chorionic gonadotrophin, human folate binding protein (LK26), alpha1 feto-protein and PAX-8.

In the broadest sense, antibody-based enrichment may utilize any technique that selects cells (i.e. positive selection) or depletes cells (i.e negative selection) according to
15   antigen expression or non-expression, as the case may be. A non-exhaustive list includes panning, complement-mediated lysis, fluorescence-activated cell sorting (FACS) and magnetic activated cell sorting (MACS).

It will also be appreciated that the aforementioned techniques may be used alone or in sequential combination to enrich fetal cells.

20   Preferred methods utilize FACS.   FACS can enrich samples by using physical cellular characteristics including but not limited to size, shape, granularity, relative DNA content and the like and/or fluorescently labelled antibodies.

Cell Filtration: Pre-filtering cell solutions before FACS analysis has two benefits, 1: Filtration allows the large scale reduction/elimination of particular sized cells and/or
25   debris. If a target cell is known to be of a particular size, these cells can be separated from solution and retained, thereby enriching their relative numbers. 2: Filtration prepares the cell sample for the purposes of FACS. It removes debris that can disrupt the flow of sample through the machine. It can also disrupt charge separation by adversely affecting the charging of individual droplets at the flow cell tip. Large debris can also be filtered out
30   of solution. Large particles/cell clumps can clog the flow cell and need to be removed. Upon the completion of filtration, cells should be resuspended in 0.1% EDTA to limit cells forming clumps. Cell filtration results are shown in Figure 7.2 – a buccal epithelial sample

was first filtered using 75?m nylon mesh and subsequently filtered using 15?m nylon mesh.

For FACS enrichment, cells of interest can be analysed, enriched and or isolated using the following parameters, either singly or in any combination:

5   Forward Scatter: Indicates the size of a cell or particle.

Size Scatter: Indicates the relative granularity of a cell or particle

DNA Content: The DNA-intercalating dye Propidium Iodide (PI) labels non-viable cells. To fluoresce, PI must be intercalated with DNA and excited by a 488nm laser. As such, the relative DNA content of a particle can therefore be ascertained by the intensity
10   of signal from DNA-bound PI.

Antibody labelling: Labelling is carried out in two stages. The first stage involves the binding of primary antibodies that target particular cell surface antigens. These antibodies are generally species specific. The second stage involves the binding of fluorochrome-conjugated secondary antibodies. These antibodies bind the specific
15   species from which the primary antibody was generated. The secondary antibody can be bound with a variety of fluorochromes that include, but are not limited to, FITC, Texas Red, PE, PerCP, PE-Cy7, PE-Cy5. When the cell-primary-secondary complex is passed through the flow cell, the laser light excites the bound fluorochrome, this signal is analysed and the cell can then be deflected for collection.

20   Samples may also undergo negative and/or selection procedures. Positive selection is where cells of interest are labelled and those labelled cells are separated from unlabelled cells and the unlabelled cells discarded. Negative selection is similar but cells of interest are unlabelled and it is the labelled cells that are discarded.

In a preferred embodiment, fetal cells positively selected and are enriched by
25   FACS using antibody to placental lactogen, placental alkaline phosphatase, PAX8 and human chorionic gonadotrophin.

In another preferred embodiment can be isolated from embryonic cells and the nuclei sorted and isolated by FACS and/or by other methods known to those of skill in the art.

30   Charge Flow Separation

Charge flow separation uses dielectrophoretic forces which occur on cells when a non-uniform electrical field interacts with field-induced electrical polarization. Depending on the dielectric properties of the cells relative to their suspending medium, these forces can be positive or negative, directing the cells toward strong or weak electrical field

5   regions. Because cells of different types or in distinct biological states have different dielectric properties, differential dielectrophoretic forces can be applied to drive their separation into purified cell populations (Wang et al., 2000. Analytical Chemistry 72 832-839).

6.2   Fetal cell enrichment

10   Fetal cells may be enriched by selective growth in the presence of appropriate cytokines and culture conditions that favor the selective proliferation of fetal progenitor cells over maternal cells. Selective growth may be performed after initial isolation or enrichment by one or more other enrichment methods.

For example, fetal nRBC's (nucleated red blood cells) may be cultured after

15   gradient enrichment and/or MACS enrichment in culture media containing many fetal NRBC growth factors (Bohmer et al., 1998, Br J Haematol 103 351-360). It is also contemplated that culture with fetal NRBC growth factors may stimulate a much higher basal proliferative capacity than mature progenitor cells and that this can be enhanced by addition of cytokine cocktails such as flt-3 ligand and thrombopoetin (Holzgreve et al.,

20   2000, Baillieres Best Pract Res Clin Obstet Gynaecol 14 709-722).

In light of the foregoing, a preferred embodiment of the invention provides a method of fetal cell isolation and analysis including the steps of:

(i)   improved high throughput enriching of fetal cells from a Pap smear sample according to physical and/or fluorescent characteristics where

25   at least one antibody binds to a fetal cell antigen.

Preferably, step (i) includes the sequential steps of:

(a)   protein digest to release cellular mixture into discrete cells

(b)   using FACS to determine and separate fetal cells by physical characteristics

30   (c)   using FACS to determine and separate fetal cells based on fluorescent characteristics using antibodies such as placental lactogen;

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(ii)   Improved high throughput amplification of nucleic acid product from pooled or single cells using a generic kit such as Genomiphi.

(iii)   Genetic identification of sample using DNA fingerprinting. Genetic identification can be undertaken using STR profiling as previously published (Findlay et al., 1997, Nature 389 355-356)

(iv)   Genetic analysis using a variety of non-limiting techniques such as RT-PCR, CGH, FISH, multiplex PCR, SNP analysis, and/or simultaneous analysis of any combination of above.

Preferably, step (iv) includes the sequential steps of:

(a)   Higher throughput genetic analysis

(b)   Detection of genetic product; and

(c)   genetic analysis, whether by manual, semi-automated or automated means. Improved higher throughput automated data processing to create analysis report. Analysis reports can include indicators of diagnosis/screening markers, disease status as well as factors such as cell origin. Automated analysis provides the capacity to analyse many millions of analyses parameters extremely quickly and thus provide high throughput analysis.

The improvements (and particularly, the combination of each of the improvements) in the above steps allow high throughput processing and analysis of embryonic cell samples

Other uses of enriched cells is for subsequent genetic analysis, biochemical analysis, morphological analysis, histology, cytology, cell culture and the like.

6.3   Genetic analysis.

A more preferred use is for genetic analysis.

As used herein, "genetic analysis" and "genetic diagnosis" are used interchangeably and broadly cover detection, analysis, identification and/or characterization of isolated genetic material and includes and encompasses terms such as, but not limited to, genetic identification, genetic diagnosis, genetic screening,

genotyping and DNA fingerprinting (also commonly known as STR profiling) which are variously used throughout this specification.

The term "nucleic acid" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNAi and DNA inclusive of cDNA, genomic DNA and DNA-RNA
5   hybrids. The nucleic acid may be contained within a cell, within the nucleus of a cell or isolated.

A "polynucleotide" is a nucleic acid having eighty (80) or more contiguous nucleotides, while an "oligonucleotide" has less than eighty (80) contiguous nucleotides.

A "SNP" is a single nucleotide polymorphism.

10   A "primer" is usually a single-stranded oligonucleotide, preferably having 12-50 contiguous nucleotides which, for example, is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as Taq polymerase, RNA-dependent DNA polymerase or Sequenase™.

15   By "genetic marker" or "marker" is meant any locus or region of a genome. The genetic marker may be a coding or non-coding region of a genome. For example, genetic markers may be coding regions of genes, non-coding regions of genes such as introns or promoters, or intervening sequences between genes such as those that include polymorphisms (such as single nucleotide polymorphisms), tandem repeat sequences, for
20   example satellites, microsatellites, short tandem repeats (STRs) and minisatellites, although without limitation thereto.

A "probe" may be a single or double-stranded oligonucleotide or polynucleotide, suitably labelled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

25   Genetic analysis may be performed by any method including, but not limited to, fluorescence in situ hybridization (FISH), primed in situ synthesis (PRINS) and nucleic acid sequence amplification, preferably in the form of multiplex fluorescent PCR amplification (MFPCR).

Examples of fluorescent in situ hybridization (FISH) and Primed In Situ Synthesis
30   (PRINS) may be found in Findlay et al., 1998, J. Assisted Reproduction & Genetics 15 257.

(a)   Multiplex fluorescent PCR

As used herein, "multiplex amplification" or "multiplex PCR" refers to amplification of a plurality of genetic markers in a single amplification reaction.

MFPCR has been shown to be a reliable and accurate method for determining sex

5   (Salido et al., 1992, Am. J Human genetics 50 303; Findlay et al., 1994a, Human Reproduction, 9 23; Findlay et al., 1994b, Advances in Gene Technology: Molecular Biology and Human Genetic Disease. Vol 5, page 62. Findlay et al., 1995, Human Reproduction 10 1005-1013; Findlay et al., 1998c, supra) diagnosing genetic diseases such as cystic fibrosis (Findlay et al., 1995, supra), detecting chromosomal aneuploidies

10   and in genetic analyses for genetic identification, such as typically referred to as DNA fingerprinting (Findlay et al., 1997, Nature 389 355-356).

With regard to genetic markers for genetic analysis, preferred genetic markers are STR and/or SNP markers. International Application PCT/AU02/01388 provides an extensive array of STR markers and primers together with MFPCR methodology to

15   successfully amplify multiple STR markers from limiting amounts of nucleic acid template.

Although from the foregoing a preferred method of genetic analysis is PCR, nucleic acid sequence amplification is not limited to PCR.

Nucleic acid amplification techniques are well known to the skilled addressee, and also include ligase chain reaction (LCR) as for example described in Chapter 15 of

20. Ausubel et al. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons NY, 1995-1999); strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252; rolling circle replication (RCR) as for example described in Liu et al., 1996, J. Am. Chem. Soc. 118 1587 and International application WO 92/01813 and by Lizardi et al., in International Application WO 97/19193; nucleic acid sequence-based

25   amplification (NASBA) as for example described by Sooknanan et al.,1994, Biotechniques 17 1077; and replicase amplification as for example described by Tyagi et al., 1996, Proc. Natl. Acad. Sci. USA 93 5395.

The abovementioned are examples of nucleic acid sequence amplification techniques but are not presented as an exhaustive list of techniques. Persons skilled in

30   the art will be well aware of a variety of other applicable techniques as well as variations and modifications to the techniques described herein.

As used herein, an "amplification product" refers to a nucleic acid product generated by a nucleic acid amplification technique.

Although the invention also contemplates use of nucleic acid other than DNA, preferably the nucleic acid is DNA.

5   More preferably, the nucleic acid is genomic DNA.

Isolation of cellular nucleic acids is well known in the art, although the skilled person is referred to Chapters 2; 3 and 4 of Ausubel et al. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons NY, 1995-1999), for examples of nucleic acid isolation.

10   (b)   SNP analysis

Single Nucleotide Polymorphisms (SNP) are the most frequent form of variation found in the genome, estimated to occur every 1000 bases. SNP genotyping has multiple applications such as predictive medicine, personal medicine, forensic identification and pharmacogenomics. SNP genotyping has already been used to investigate a number of

15   disorders such as cystic fibrosis, Factor V Leiden mutation, and factors such as A, B, O and Rh blood grouping. However conventional SNP analysis is limited by the relatively high amount of extracted DNA usually required (up to 100ng) for analysis. However in genomic analysis, there is increasing demand to both maximize data by performing multiple analyses and secondly to analyze minimum amounts of sample, even to the

20   single cell level. Although multiplex SNP analyses can be performed routinely using a variety of techniques such as homolgous mass extension, multiplex single cell SNP analysis is still problematic. Although multiplex single cell SNP analysis has been published (Findlay et al, 2003, Todays Life Sciences 15 (5) 34-36), these techniques are not amenable to high throughput processing. This invention also anticipates that

25   improvements to increase sample throughput and SNP processing will be combined with techniques and methods mentioned herein to provide improved SNP analysis from reproductive tract cells.

Preferred sources of nucleic acids are mammals, preferably humans.

The invention also contemplates genetic analysis of non-human samples such as

30   from cows, sheep, horses, pigs and any other mammal including companion animals, sporting animals and livestock, although without limitation thereto.

So that the invention may be readily understood and put into practical effect, reference is made to the following non-limiting examples.

(c)   Single Cell Whole Genome Amplification (WGA)

Single Cell WGA has been attempted using several strategies with limited
5   success. While genomic DNA has been amplified from single cell sources, balanced representation of the DNA from single cells has been very difficult to achieve due to the very high levels of allelic dropout, whole locus dropout and preferential amplification.

This can be attributed to several factors, which may include; incomplete lysis of the cell before WGA, the properties and quality of the enzyme used;   the melting
10   behaviour of genomic DNA not being accounted for during amplification, incomplete amplification etc. The effect of uneven representation of the genomic DNA can have very serious even critical consequences on downstream applications, including MF PCR which could result in misdiagnosis or misidentification.

Particular embodiments of this invention include significant improvements such as
15   improved lysis protocols and the use of particular enhancements to control the characteristics of the nucleic acid during amplification, particularly when combined with commercial whole genome amplification kits such as GE Healthcare Genomiphi kit – utilising the Phi29 DNA polymerase which has very high processivity and strand displacement properties or the Rubicon Genomics GenomePLex kit.

20 7 .   MATERIALS AND METHODS

7.1   Fetal cell Isolation

Informed consent was obtained from pregnant women between 7 and 31 weeks gestation. A pap smear cervix brush (Rovers Medical Devices, Lekstraat, The Netherlands) was inserted through the external os to a maximum depth of two
25   centimeters. The brush was then removed whilst rotating a full turn. The material that was caught on the brush was smeared on a slide which was used for routine cervical cancer screening. The remaining material on the brush was included in the study. Some pregnant women were undergoing pregnancy termination immediately after the pap smear sample was taken, and in these cases the pap smear sample was not used for
30   routine cervical screening and the entire sample was available for analysis.

Cells were washed off the cervix brush into Dulbeccos PBS (Invitrogen, Melbourne

Australia). The cells were then spun at 402g in a Sigma 4K15 centrifuge (Sigma, St Louis, USA), the supernatant was poured off and the remaining material transferred to a 1.5mL centrifuge tube. The cells were then spun at 3000rpm in a biofuge pico (Kendro, Ashville, North Carolina), the supernatant was then poured off and the cells were
5　resuspended in 800uL PBS. A buccal swab was also taken from the mother to provide an uncontaminated source of maternal cells.

7.2　Trypsin Digestion

Samples were treated to disassociate cell clumps into single cell suspensions using techniques well known in the art such as trypsin digestion.

10　　　-　3 X 200uL + 1 X 50uL cell suspension from each sample

-　200uL (or 50uL) 2.5% Gibco Trypsin/EDTA in PBS was added

-　Incubated 37 degrees for 15-30 minutes

-　1mL PBS was added and then centrifuged 3000rpm 5 minutes (Biofuge pico)

15　　　-　Supernatant was removed

50uL initial material sample was placed in -20°C freezer for later analysis

7.3　Isolation of fetal cells by physical characteristics using FACS

o　Cells should be washed 2-3x with FACS buffer (PBS supplemented with either 1% BSA or 5% FBS and containing 0.05% NaN3).

20　　　o　Suspend the cell pellet from the final wash in 50 microliters FACS buffer (or more if more than one analysis is to be done on a single sample).

o　Incubate for 30 minutes on ice.

o　Wash cells 2-3x with FACS buffer and suspend in 200-300 microliters
25　　　　　FACS buffer for analysis.

Fetal cells may be isolated by any of the aforementioned cell isolation methods.

In all cases samples from non-pregnant women are run as control cases to determine the base-line level of non-specificity.

Preferably, said one or more fetal cells are isolated by FACS sorting.

Said one or more fetal cells may be isolated from any pregnant mammal.

5   Preferably, said one or more fetal cells are isolated from a pregnant human.

When a fetus is at increased risk for genetic defects such as chromosomal anomalies, prior art prenatal diagnosis is by invasive procedures such as either chorionic villus sampling (CVS) in the late 1st trimester or amniocentesis in the 2nd trimester of pregnancy. By the third trimester, a combination of CVS and amniocentesis, or even fetal 10 blood sampling, may be necessary.

· A rapid, less-invasive and low cost method of prenatal diagnosis involves genetic diagnosis from fetal cells shed into the cervical sump at 6-20 weeks of gestation. These samples are obtained from the cervix by cytobrush in a manner identical to a PAP smear, which is similar to but significantly less invasive than invasive transcervical sampling.

15   Although promising, previous work has identified several major difficulties. Firstly the need to obtain the large numbers of fetal cells required for genetic diagnosis. Secondly the isolation of fetal cells from the cervical sample is extremely difficult as recent results suggest that fetal cells could be isolated and diagnosed in only ~22% of cases due to the presence of "contaminating" maternal cells. Previous approaches have 20 generally concentrated on isolating fetal cells by morphology or cell sorting.

Unfortunately, morphology grading is extremely time-consuming, expensive and generally unreliable and inaccurate. Alternative cell sorting techniques involves antibody-labelled slides to capture fetal cells, which is generally unspecific resulting in major maternal contamination and misdiagnosis or insufficient fetal cells. Thirdly the difficulty in 25 positively identifying the isolated cell as being fetal rather than maternal; previous approaches have determined male fetal cells which identifies the fetus as the mother is female but female signals could either indicate female cells or maternal contamination and thus misdiagnosis.

Fourthly the difficulty of obtaining genetic diagnosis from small cell numbers. Although fetal cells have been identified in cervical samples (mainly by identifying male cells within the sample) aneuploidy screening (the primary reason for prenatal diagnosis) cannot usually be performed nor diagnosis made if the fetus is female due to the risk of

5   contamination causing misdiagnosis.

Finally, although PAP sampling collection is theoretically much safer than CVS and amniocentesis as PAP smears have been taken during pregnancy for many years, relative safety remains to be fully evaluated.

According to the present invention, it is preferred that said fetal cells are present in

10   a maternal uterine cavity or endocervical canal sample, particularly a transcervical sample. Methods of isolating fetal cells include cervical cotton swab, cytobrush, aspiration of cervical mucus, lavage of the endocervical canal and uterine lavage. Samples can be obtained from transcervical aspiration of mucus from just above the internal os or the lower uterine cavity. Lavage is generally conducted with a saline wash, but other isotonic

15   solutions are suitable. Typically, endocervical lavage with 5-10ml or intrauterine lavage with 10-20ml saline provides sufficient fetal cells upon separation from maternal cells. The sample may be collected using a combination of methods.

Preferably, cell samples are isolated from a female human in the first trimester of pregnancy or when the fetus is between 6 to 17 weeks gestation. The sample can be in

20   any solution which maintains cell integrity and minimizes cell lysis or damage, preferably a physiological solution, or more preferably, a saline solution or tissue culture medium with or without the addition of sera.. The sample is preferably stored at 0°C to 4°C until use to minimize the number of dead cells, cell debris and cell clumps.

Preferably, to aid fetal cell separation, clumps of cells are preferably treated to

25   obtain a suspension of single cells. The clumps may be separated by techniques known to a skilled person, such as enzymatic, chemical or mechanical separation. For example, enzymatic separation may utilise protease or trypsin. Chemical separation may utilise acetyl cysteine and mechanical separation may involve gentle teasing, aspiration or micromanipulation.

30   The number of fetal cells in the sample varies depending on factors including the age of the fetus, method of sampling, number and frequency of samplings, the vigour of sampling and the volume aspirated.

Maternal uterine cavity or endocervical canal samples typically contain at least two main types of nucleated fetal cells: cytotrophoblasts and syncytiotrophoblasts cells.

Fetal cells can be isolated either by selecting fetal cells from maternal cells (positive selection) or isolating the maternal cells from the fetal cells (negative selection) or most preferably a combination of both. Preferably, the nucleated fetal cells are retained in the purified sample.

Preferably, for negative selection the cells are labelled with an antibody for a common cell type antigen that should not be significantly expressed in fetal cells. The negative selection procedures sorts those cells that are labelled with the negative selection antibody and retains the unlabelled cells. The unlabelled cells will consist of fetal cells as well as maternal cells that may not express the antigen or fail to bind to the antibody, hence the reason for serial selections. .

Suspension media, use of protein supplements such as 5-10% BSA or HSA and appropriate cell concentrations for FACS-based separation are well known in the art and described in references such as Practical Flow Cytometry supra and Current Protocols in Immunology Eds Coligan et al., supra.

More preferred as a protein supplement is about 5% autologous plasma, which can be harvested from a purified blood sample and is non-immunogenic.

The cells of the sample, preferably purified cells, are labelled with fluorescent antibodies specific for the antigens encoded by at least one maternal locus, selected as described previously. The antibodies can be polyclonal or monoclonal, preferably monoclonal. Preparation of polyclonal and monoclonal antibodies for an antigen of interest is well known. Furthermore, there is a vast supply of potentially useful antibodies, such as to human HLA antigens, that are commercially available or available from hybridoma depositories such as the ATCC.

For example, in the case of separation using HLA antigens, HLA antigen-specific antibodies are commercially available. Typically the HLA Class 1 loci (A, B and C) and the Class 11 DR and DQ loci are determined by serological methods. Therefore, antibodies specific for those antigens are readily available. Sources of HLA antigen-specific antibodies include Genetic Systems (Seattle, Wash.) and C6 Diagnostics (Mequon, Wis.). Blood group antigens are also determined serologically and the antibodies are commercially available.

The antibody is labelled with a dye that facilitates, cell sorting, particularly a fluorochrome, Suitable dyes for FACS analysis and/or separation are well known in the art. Examples of dyes are described in Practical Flow Cytometry (Second Edition), supra, at pages 11 5-198 and in Chapter 5 of Current Protocols in Immunology, supra. Preferred

5 . dyes are fluorochromes including fluorescein (e.g., fluorescein isothiocyanate–FITC), rhodamine (e.g., tetramethylrhodamine isothiocyanate–TRITC), phycoerythrin (PE), allophycocyanin (APC) and Texas Red (Molecular Probes, Eugene, Oreg.).

For four-color flow cytometric sorting, cells can be labelled with antibodies for antigens expressed by four alleles. In that case, preferably, the antibodies are specific for

10 . both antigens expressed by the alleles of two maternal HLA loci. Maternal cells are labelled with all four fluorochromes. Fetal cells are labelled with two of the four fluorochromes when none of the nontransmitted maternal alleles is inherited from the . father. By using four fluorochromes from two loci, the fetal cells remain distinguishable from the maternal cells even when the fetus inherits one of the nontransmitted maternal

15   alleles from the father. A second staining is only necessary when the fetus inherits both · nontransmitted maternal alleles from the father. When the antibodies are for antigens expressed by three or four maternal loci, using the additional dyes increases the likelihood that the fetus did not inherit each of the maternal alleles. ʼ

Fetal cells are only indistinguishable from maternal cells by the method of the

20   present invention in the case where the fetus inherits all six non-transmitted maternal · alleles from the father.

For antibody labelling, cells are preferably incubated at about 4°C to maintain cell integrity. Incubation for about 30 minutes at 4°C is usually sufficient for substantially complete antibody binding. The sample is preferably mixed, as by using a hematology

25   blood rocking device, during the incubation to ensure .contact of the antibodies with the · · cells. Preferably, the incubation is performed in the dark when using a fluorochrome label. Secondary reactions (e.g. incubation of fluorochrome-labelled avidin with biotin labelled cells) are performed in the same manner.

In a preferred embodiment, an additional selection criterion is DNA content. Fetal

30   cells having greater than 2C DNA content can be determined using a number of vital- · staining fluorochromes such as the Hoechst dyes, DAP1 (4-6-diamidino-2-phenylindole), hydroethidine and 7-aminoactinomycin D (7AMD). The fluorochrome used depends on the labels used to select the fetal cells. A second laser capable of emitting UV light is required to excite Hoechst and DAPI dyes. Each of the above-described dyes can be

used with FITC and PE.

The ability of the cell sorter to separate maternal and fetal cells ultimately depends on the percentage of fetal cells in the sample. To obtain a fetal cell sample that is at least about 60% pure (60% of the sorted cells are fetal cells), the fetal cells should
5   constitute about 0.001% of the maternal cells or greater. Preferably, the sample contains 80%, more preferably 90% fetal cells post-sorting.

When 100% purity is desired, the sorted cells can be plated for subsequent analysis. For example, cell suspensions containing an individual cell can be isolated within a preselected volume of suspension medium by limiting dilution. Drops containing
10   individual cells can then be placed in suitable pre-made containers (e.g. 96 well plates) for subsequent nucleic acid amplification and/or analysis.

For PCR analysis, analysis can be performed using a single, unambiguously identified fetal cell, identified for example by DNA fingerprinting.

Alternatively, ways can be envisaged of identifying monozygosity (indicative of the
15   presence of a monogenic disease) in a mixed cell population containing minimal fetal material including as few as one fetal cell in ten cells. Following sorting, the separated cells can be washed twice in a physiologic buffer and resuspended in an appropriate medium for any subsequent analysis to be performed on the cells.

In addition, it is envisaged that enriched cells may also be tested for particular
20   genetic markers not present in the maternal samples such as Y-chromosome markers if the embryos is male. However the use of such techniques are limited when the gender of the embryo is not previously known.

Following the present recovery method, whether based on solid phase (eg. magnetic beads or panning) or FACS separation, the fetal cells can be used in the same
25   manner as fetal cells obtained by other methods such as anmiocentesis and chorionic villus biopsy. The cells can be used as a source of DNA for analysis of the fetal alleles, as by polymerase chain amplification. PCR analysis methods may be used to detect, for example, fetal sex, beta thalassemia, phenylketonuria (PKU), and Duchennes muscular dystrophy.

30   Alternatively, the cells can be cultured in the same manner as biopsy materials for karyotyping analyses. However, the incubation period may be significantly shortened if a DNA content of greater than or equal to 2C is used as a selection criterion.

7.4   Isolation of antibody labelled fetal cells by FACS

An example of fetal antibody protocol is described, although variations are contemplated.

Samples were placed through the following protocol:

5   •   10 minutes in 10% Donkey serum (Sigma-Aldrich, St. Louis, USA) diluted in PBS

        •   Donkey serum was removed

        •   100uL of each of the following Primary Antibodies, 1/100 dilution in 10% donkey serum (i.e. final dilution 1/200) was then added.

10   •   ab7816 Rabbit anti-human chorionic gonadotrophin antibody (Abcam, Cambridge, UK) and NCL-PLAP Mouse anti- placental alkaline phosphatase antibody (Novocastra Laboratories, Newcastle, UK).

        •   Samples were then incubate 90 minutes at room temperature in humidified chamber

15   •   Samples were then washed 3 X 5 minutes in PBS

        •   100uL of each following Secondary antibody 1/200 dilution in PBS (ie final dilution 1/400) was then added.

        •   Donkey FITC polyclonal to rabbit IgG (Abcam, Cambridge, UK) and Chicken Rhodamine polyclonal to mouse IgG (Abcam, Cambridge,
20   UK).

        •   Samples were then incubated 45 minutes at room temperature in humidified chamber

        •   Samples were then washed 2 X 5 minutes PBS

        In all cases samples from non-pregnant women are run as control cases to
25   determine the base-line level of non-specificity.

        To improve the accuracy, reliability and cost effectiveness of non-invasive prenatal genetic diagnosis from pap smears, it is necessary to develop enrichment strategies that

both reduce the concentration of contaminating cells and secondly recover as many fetal cells as possible. The use of well known digesting enzymes such as trypsin or collagenase increase the number of fetal cells retrieved.

In one embodiment, genetic analysis of fetal cells isolated from pap smears preferably requires a number of serial enrichment strategies in order to provide a reliable source of relatively uncontaminated fetal cells. Initial FACS enrichment strategies usually identify cells using physical characteristics such as density, charge or size. Although single cycles are not highly specific they do reduce target cell loss and are relatively low in cost, therefore multiple cycles, either on physical or fluorescent characteristics, are utilised to maximise specificity whilst maintaining cellular yield. Secondary enrichment strategies such as antibody staining will often identify cells using specific cellular traits. Primary and secondary enrichment strategies work in unison to provide a reliable source of uncontaminated fetal cells yet achieve maximum yield. Performing multiple cycles to improve purity and/or yield are not significant time or sample limiting steps.

7.5   Cell processing with FACS

Cells were scanned by the Altra flow cytometer (Beckman Coulter) and the resultant data analysed by the accompanying Altra EXPO 32 Multicomp analysis software (Beckman Coulter), using combinations of analysis parameters of forward scatter, side scatter, relative DNA content and antibody labelling data. Cells of interest were determined from two-dimensional scatter graphs and gated in the Multicomp software. The instrument was then directed to sort individual cells within these gates into a 96-well plate containing 1?l of Lysis Buffer (200mM potassium hydroxide/50mM Dithiothretol, 0.001% SDS) in each well using the Autoclone module. Immediately upon completion, the plate was sealed with a Qiagen Tape Pad, spun down in a plate centrifuge and incubated at 65°C for 10 minutes. 1?l neutralising buffer (200mM HCl/200mM beta-mercaptoethanol) was then added. Cells were stored at –20°C until MFPCR.

7.6   Genetic identification using DNA fingerprinting

Multiplex fluorescent PCR of Amelogenin and STRs listed in Table 1 or more preferably listed in Table 3, D3S1358, D5S818, D7S820 and CSF1P0 THO, D21S11, D18S51, VWA, FGA, D3S1358, D5S818, D7S820, CSF, TPOX was performed on isolated cells. Each reaction contained forward and reverse primers, 1 X PCR buffer (Applied Biosystems, USA), 1.5mM $MgCl_2$ (Applied Biosystems, USA), 1.25mM each dNTP (Gibco, Life Technologies, Melbourne, Australia) and 1 unit HotStart Taq (Qiagen,

Australia). PCR conditions were 94°C for 2 minute denaturation followed by 45 cycles of 94°C/10 second denaturation, 57°C/1 minute annealing and 68°C/30 second extension.

PCR product was processed using Ammonium.acetate/Ethanol Clean-up. Post clean-up processing involved adding 2uL of cleaned-up product to 3uL loading buffer
5   (Amersham Biosciences, Piscataway, New Jersey). Samples were then heated to 90 degrees for 60 seconds and placed immediately on ice. Analysis was completed using the Megabace 1000 capillary electrophoresis system with Genetic Profiler Version 1.5 software (Amersham Biosciences, Piscataway, New Jersey). Injection parameters were – 3kV for 45 seconds and run parameters were –10kV for 75 minutes at 44°C.

10   The procedure to identify a fetal signal within that produced by MFPCR of the isolated cells was that outlined in the paper 'Analysis and interpretation of mixed forensic stains using DNA STR profiling' (Clayton et al., 1998, Forensic Science International 91 55-70). That is the STR is an additional band to that found in the maternal fingerprint i.e. consistent with maternal signal. It is not consistent with a stutter band or artefact peak
15   and that it is the same base pair size as bands identified as fetal for the same locus within other isolations from the same patient.

Protocols for enrichment and diagnosis of fetal cells from the cervix must be consistently successful, robust and inexpensive if the techniques are to become an alternative to invasive procedures such as amniocentesis or chorionic villus sampling.
20   Previous inventions and work has been significantly limited by a variety of factors including: obtaining sufficient cells; isolation of fetal cells from the sample; genetic identification of cells to determine fetal source; genetic diagnosis from small cell numbers and sample collection.

Where previous work has failed to detect and analyse fetal cells at a consistent
25   and reliable enough level to be considered as a promising tool towards minimally invasive prenatal diagnosis, this invention provides incremental improvements to multiple steps and combines them into a high throughput method allowing widespread application for the first time.

However alternatively it should also be appreciated that this invention may also be
30   considered as a complementary technique to other non-invasive or minimally invasive tests such as biochemical screening and ultrasound screening offered to pregnant women during the first trimester of pregnancy (Daryani et al., 2000, J. Obstet. Gynecol. 183 752).

Our results indicate that cells reacting against multiple antibody sets are present in all patients. The percentage of fluorescent cells vary, with no apparent correlation to gestation. The overall number of fluorescent cells in the sample also varies with no correlation to gestation. This may indicate that variation in the number of fetal cells in the
5   sample is specific to the patient or perhaps more likely due to variations in the technique used by the operator performing the retrieval.

DNA fingerprinting using MFPCR was used to confirm cell origin of the fluorescent cells from each antibody set and patient. This MFPCR technique has the advantage of being highly discriminating for cell origin even when applied to very close relatives such
10 as mother and baby.

Combining the antibody and MFPCR data from all patients, it is possible to determine a minimum specificity for each antibody set towards fetal cells although this may be due to non-specific binding to non-cellular particles, binding to non-intact cells or strong binding to a maternal cell lineage..

15   7.7   STR Genetic analysis

Previous work using genetic diagnosis of limited numbers of fetal cells obtained from the uterine cervix using techniques such as FISH and PCR is very limited (methods are compared in Table 4). FISH analysis can only identify fetal cells if they are aneuploid or originate from a male fetus (Fejgin et al., 2001, supra) – this is an important and
20 considerable limitation to the use of such techniques for prenatal diagnosis. Other studies use PCR analysis to detect disorders however in most cases this is limited to the gene analysed and quantitative variations in the maternal and fetal alleles, for example RH(D) analysis (Tutschek et al., 1995, Prenatal Diagnosis 15 951). Again this is an important and considerable limitation to the use of such techniques for prenatal diagnosis.

25   However MFPCR has the advantages of overcoming these limitations, as it is not limited by sex or individual gene alleles. MFPCR has an extremely high level of discrimination between closely related individuals, can be performed on single cells and provides multiple diagnoses within a single reaction.

In this embodiment, MFPCR was used to accurately determine the presence of
30 fetal cells in a mixed fetal/maternal sample. For these reasons we suggest that MFPCR be considered the preferred method of choice when performing prenatal genetic diagnosis from pap smear samples.

7.8   Single Cell Lysis Protocol:

To ensure complete lysis of fetal cells, the lysis protocol was improved as follows to maximise DNA accessibility :

5

- . 1ml of Lysis Buffer (200mM KOH, 50mM DTT, 0.001% SDS) is added to a single cell in a 0.2ml tube or plate.

- . Incubate at 65°C for 10 minutes.

- 1ml of Neutralising Buffer (200mM HCl, 200mM beta-mercaptoethanol) is added to the lysate.

- Tubes or plates are then stored at –20°C until needed.

10   Multiplex fluorescent PCR of Amelogenin and STRs D3S1358, D5S818, D7S820, CSF1P0, THO, FGA, D21S11, and D18S51 or any markers listed in Table 1, or more preferably Table 2, was performed on isolated cells using previously described protocols . (Findlay et al., 2001, supra; International Application PCT/AU02/01388). Each reaction contained forward and reverse primers, 1 X PCR buffer (Applied Biosystems, USA),
15   1.25mM each dNTP (Gibco, Life Technologies, Melbourne, Australia) and 1 unit Qiagen HotStarTaq (Qiagen Melbourne, Australia). PCR conditions were 94°C/2 minute denaturation followed by 45 cycles of 94°C/10 second denaturation, 57°C/1 minute annealing and 68°C/30 second extension. The PCR uses no oil overlay, as the heated lid of the PCR is sufficient. The PCR is taken off the block and stored at 4°C until required
20   for electrophoresis.

PCR product was processed using Ammonium acetate/Ethanol Clean-up. Post clean-up processing involved adding 2uL of cleaned-up product to 3uL loading buffer (Amersham Biosciences, Piscataway, New Jersey). Samples were then heated to 90° degrees for 60 seconds and placed immediately on ice. Analysis was completed using the
25   Megabace 1000 capillary electrophoresis system with Genetic Profiler Version 1.5 software (Amersham Biosciences, Piscataway, New Jersey). Injection parameters were – 3kV for 45 seconds and run parameters were –10kV for 75 minutes at 44°C.

The procedure to identify a fetal signal within that produced from MFPCR of the isolated cells was that outlined in Clayton et al., 1998, supra.

30   This embodiment also indicates that cells of fetal origin are indeed present in the endocervical canal of the mother. Due to the relatively low number of fetal cells present

serial enrichment strategies must be utilized to in unison to provide a reliable source of uncontaminated fetal cells yet still provide maximum yield. Once a reliable source of fetal cells is established, the diagnostic techniques such as MFPCR used to screen these cells needs to not only confirm fetal origin and but also test for genetic traits.

5   Again MFPCR has the advantage of being highly discriminating for cell origin even when applied to close relatives such as mother and baby. MFPCR can be performed on single cells and provides multiple diagnosis within a single reaction. In this embodiment MFPCR was used to accurately determine the presence of fetal cells isolated from a mixed fetal/maternal sample.

10   This embodiment demonstrates that samples highly enriched in fetal cells (>90%) can be produced even though an uncontaminated source of fetal cells from pap smears (i.e. isolation of 100% fetal cells) may not be possible. Single fetal cells can then be easily isolated and used to screen for genetic traits. For this reason, and the ability to test for multiple probes, MFPCR may be considered the method of choice when performing
15   prenatal genetic diagnosis from pap smear samples.

7.9   SNP genetic analysis

Many common diseases in humans are not caused by variation within single genes but are instead influenced by complex interactions among multiple genes as well as a multitude of environmental and lifestyle factors. Genetic factors may also confer
20   susceptibility or resistance to a disease as well as determine the severity or progression of the disease. As most of the factors involved in these intricate pathways are unknown, it has therefore been difficult to develop screening tests for many diseases and disorders. It is therefore vital to understanding the genetic basis of common human diseases and this depends on a detailed understanding of the variability observed in the human genome.

25   Single Nucleotide Polymorphisms (SNP) are the most frequent form of variation found in the genome, estimated to occur every 1000 bases. SNP genotyping has multiple applications such as predictive medicine, personal medicine, forensic identification and pharmacogenomics However conventional SNP analysis is limited by the relatively high amount of extracted DNA usually required (up to 100ng) for analysis. However in genomic
30   analysis, there is increasing demand to both maximize data by performing multiple analyses and secondly to analyze minimum amounts of sample, even to the single cell level. Although multiple SNP analyses can be performed routinely, the degree if sensitivity is still far from single cell level analysis. Multiplex single cell SNP analysis has been

problematic and again is not amenable to the high throughput processing required of clinical application.

SNP genotyping can be used to identify genetic regions associated with a disease phenotype, allowing researchers to target particular areas of interest and begin to reveal
5   relevant genes associated with a disease. SNP patterns from a large group of affected individuals can be compared to those of unaffected individuals. These association studies can detect differences in the SNP patterns of the two groups, thereby indicating potentially important SNPs and thus genetic regions for further study. Eventually SNP profiles that are characteristic of a variety of diseases will become established. Defining
10 and understanding the role of genetic factors in disease will also allow researchers to better evaluate the role that non-genetic factors - such as behaviour, diet, lifestyle, and physical activity - have on disease.

SNP genotyping has already been used to investigate a number of disorders such as cystic fibrosis, Factor V Leiden mutation, and factors such as A, B, O and Rh blood
15 grouping.

In general SNP genotyping is undertaken in six main stages: PCR, Post-PCR cleanup, SNP primer extension reaction, final cleanup, SNP product sizing and analysis.

To provide an example of SNP genotyping we have utilised methods based around Amersham Megabace 1000 SNP manufacturers protocols.

20   (a)   PCR for SNPs

Isolated fetal cells were processes in a multiplex SNP reaction consisting of oligonucleotides for specific SNPs such as Kell, Rh etc. Each 25µl reaction contained 25pmol forward and reverse primers, 1 X PCR buffer, 5nM each dNTP (Gibco, Life Technologies, Melbourne, Australia) and 1 unit Qiagen HotStarTaq (Qiagen, Melbourne,
25 Australia). PCR conditions were 95°C for 15 minute denaturation followed by 45 cycles of 20 secs at 94°C, 60°C then 72°C then followed by a two minute extension at 72°C.

Single cell SNP PCR was also carried out. For this, the proprietry GMS Lysis Protocol was used as follows:.

Single Cell Lysis Protocol: To ensure complete lysis of cells, the following lysis
30 protocol is used:

- 1ml of Lysis Buffer (200mM KOH, 50mM DTT, 0.001% SDS) is added to a single cell in a 0.2ml tube or plate.

- Incubate at 65°C for 10 minutes.

- 1ml of Neutralising Buffer (200mM HCl, 200mM beta-mercaptoethanol) is added to the lysate.

Tubes or plates are then stored at –20°C until needed for normal SNP PCR.

(b)   Post PCR cleanup

Post PCR cleanup removes excess dNTPs and residual primers before primer extension and commonly uses SAP (shrimp alkaline phosphatase) and EXOI (exonuclease I) protocols.

Using a half-reaction protocol, 10µl of PCR product is added to 2.5µl EXO SAP (Amersham Biosciences) and incubated at 37°C for 15 minutes.

(c)   SNP primer extension reaction

Combine 1.0 µl SNuPe premix and 1µl dilution buffer (Amersham Biosciences) with 2 pmol extension primer, 1-10 ng cleaned PCR template (~1 ul) with distilled water to bring up to a final volume of 5µl. PCR conditions were 25 cycles of 96°C for 10 sec, 50° C for 5 sec then 60° C for 10 sec.

(d)   Final cleanup

The purpose of a final clean-up step is to remove excess terminators and desalts the samples prior to electrokinetic injection. AutoSeq96 columns (Amersham Biosciences) are used as per following protocol.

Spin product at 910g for 5 minutes, add 100µl deionised water, repeat spin, add samples to spin columns and repeat spin.

It is recommended to have at least one additional water wash to obtain sufficient yield good signal intensities. Additional water washes will further increase the signal intensity.

(e)   SNP product sizing

·Add 2.5ul multiple injection marker ·(MIM, Amersham Biosciences) to 497.5ul loading solution then dispense 5µl into each well. Load LPA matrix (Amersham, Biosciences) and rerun as per manufacturers protocol. Perform cycles of sample injection then two-minute electrophoresis interval and repeat upto twelve times.

5   Enter sizing and SNP parameters into Snupe and Instrument Control Manager. Analysis is performed using SNP Profiler as per manufacturers protocols.·

Single Cell Whole Genome Amplification

Single cell is picked into a 0.2ml tube or 0.2ml well of a 96-well plate. Lysis is carried out as previously described. To the ~2?l of lysate, 1?l of enhancer (such as 5M
10   Betaine) and 9?l of Genomiphi (GE Healthcare) Sample Buffer is added and heated to 95°C for 3 minutes then cooled to 4°C on ice. 9l of Genomiphi Reaction Buffer and 1?l of Genomiphi enzyme is then added to the lysate/sample buffer mix. The tube is then incubated at 30°C for 18hrs, followed by a 10 minute 65°C step to denature the enzyme. · The reaction product is then cleaned using Ammonium Acetate/Ethanol precipitation.
15   Product is re-suspended in 20l and 2l is taken for downstream reactions. Results of single cell-Genomiphi are shown in Figure 7.4 Discussion

. This specific example of the invention demonstrates that for the first time, efficient enrichment of embryonicl cells from Pap smears can be performed using an improved · · method (and particularly, a combination of improved methods), including FACS, nucleic
20   acid amplification, genetic analysis, and the other techniques discussed earlier. The combinations of this technology with improved automated procedures for genetic identification and analysis, have been applied to create an improved method which allows automated high throughput system to maximize cost effectiveness and thus offer the first practical non-invasive prenatal analysis application.

25   The cost advantages that the inventors anticipate will arise from the use of the invention include:

•   The method lends itself potentially to a high degree of automation, thereby reducing the need for human involvement in performing those aspects which are capable of being automated;

30   •   The increased speed of performing the method (through (A) reduced turnaround time for an analysis and (B) increased "throughput" [ie, the increase in the number of samples that can be processed in any ·

given time]) means that the cost of analysing a sample in any given period of time is reduced;

• Automation of the analysis steps entails a reduction in aspects of the method that are labour intensive and which involve labour related expenses;

• Those aspects of the analytical method which can be automated generally will result in a lower risk of error, and thus, the need to repeat analyses will be reduced (with a concomitant saving in the cost of consumables used in the method);

• The cell samples to be analysed can be obtained with a high degree of purity: this will result in (amongst other things), savings in the use of consumables, as the (unwanted) analysis of non-target cells will be reduced;

• The method entails the possibility of integrating a DNA fingerprinting system and a diagnostic system, which reduces the need for multiple testing of subjects;

• The method entails the potential for conducting multiple tests simultaneously on a subject. This avoids the need to test a single subject more than once.

The inventors believe that this embodiment therefore represents a substantial advance compared to prior art and confirms that non-invasive prenatal diagnosis from pap smears can be automated to provide the high through capability required for clinical application.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned in or evident from the text of this specification or the accompanying drawings. All of these different features and combinations constitute various alternative aspects of the invention, the scope of which is defined by the appended claims.

All patent and scientific literature, computer programs and algorithms referred to in this specification are each specifically incorporated herein by reference in their entirety.